PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/355, 31/35	A1	(11) International Publication Number: WO 98/38993
		(43) International Publication Date: 11 September 1998 (11.09.98)

(21) International Application Number: PCT/US98/03470

(22) International Filing Date: 23 February 1998 (23.02.98)

(30) Priority Data:
60/039,790   4 March 1997 (04.03.97)   US

(71) Applicant: WISCONSIN ALUMNI RESEARCH FOUNDA-TION [US/US]; P.O. Box 7365, Madison, WI 53707–7365 (US).

(72) Inventor: ELSON, Charles, E.; 7 Gray Birch Trail, Madison, WI 53717 (US).

(74) Agent: HAAS, George, E.; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202–4497 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF SUPPRESSING TUMOR GROWTH WITH COMBINATIONS OF ISOPRENOIDS AND STATINS
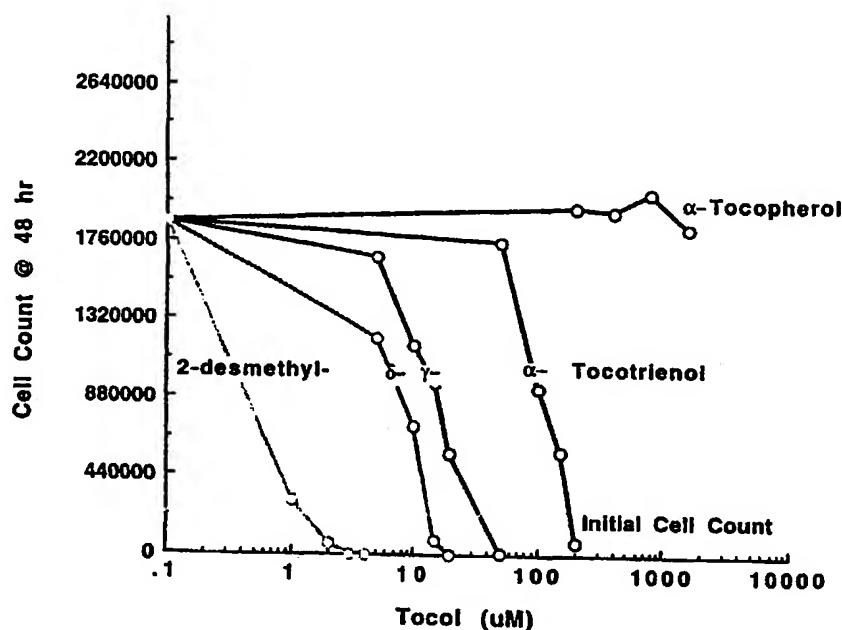
(57) Abstract

A method of inhibiting the growth of tumor cells is disclosed. In one embodiment, this method comprises the step of exposing tumor cells to an effective amount of a composition comprising at least two compounds selected from the group consisting of tocotrienols, statins and ionones.

METHOD OF SUPPRESSING TUMOR GROWTH WITH COMBINATIONS OF ISOPRENOIDS AND STATINS

Background of the Invention

Sundry mevalonate-derived constituents (isoprenoids) of fruits, vegetables and cereal grains suppress chemically-initiated carcinogenesis. This action has been attributed to

5   the isoprenoid-mediated induction of detoxifying activities and to the antioxidant activity of some isoprenoids. Neither action explains the potent impact isoprenoids have on the promotion/progression stage of chemically-initiated carcinogenesis and on the growth of chemically established and

10   implanted tumors (reviewed by Elson, 1995; Elson and Yu, 1994). Isoprenoids differ substantially in the impact they have on tumor growth. Isoprenoids suppress, via post-transcriptional actions (Correll, et al., 1994; Parker, et al., 1993; D.M. Peffley and A.K. Gayen, personal

15   communication), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, the activity deemed to be rate-limiting for the synthesis of cholesterol. The statins are competitive inhibitors of HMG-CoA reductase. Correlations between the late stage tumor-suppressive potency of diverse

20   isoprenoids and their impact on HMG-CoA reductase activity approach unity. The reductase activity of tumors differs from that of liver in being resistant to sterol feedback regulation. The tumor activity however retains high sensitivity to post-transcriptional regulation as triggered by

25   diverse isoprenoids. As a consequence of the isoprenoid-mediated suppression of HMG-CoA reductase activity the pools of mevalonate pathway intermediates become limiting for the post-translational processing of growth-associated proteins (reviewed by Elson, 1995; Elson and Yu, 1994).

30   One recent review presented a list of structurally diverse isoprenoids with varying capacity to suppress mevalonate synthesis (Elson, 1995).

Summary of the Invention

In one embodiment, the present invention is a method for

35   inhibiting tumor cell growth. The method comprises treating

-2-

the cell with a combination of at least two products of the
mevalonate pathway selected from the group consisting of
statins, ionones and tocotrienols.

In one preferred embodiment, the ionone is selected from
5   the group consisting of β-ionone; 6-10-dimethyl-undec-3,5-ene-
2,9-dione; 6,10-dimethyl-9,10-epoxy-undec-3,5-ene-2-one; 9,10-
diacetoxy-6,10-dimethyl-undec-3,5-ene-2-one; 6,10-dimethyl-
9,10-diol-undec-3,5-ene-2-one and α-ionone. Preferred
tocotrienols include d-γ-tocotrienol, 2-desmethyltocotrienol,
10   d-δ-tocotrienol and d-tocotrienol. Preferred statins include
lovastatin, pravastatin, simvastatin and fluvastatin.

In another form, the present invention is a pharmaceutical
composition for treating or preventing tumors comprising
effective amounts of at least two agents selected from the
15   group consisting of tocotrienols, statins and ionones.

It is an object of the present invention to prevent or
reduce tumor growth and metastasis.

It is another object of the present invention to increase
the duration of survival of a tumor patient following
20   detection of tumor.

It is another object of the present invention to prevent
tumor formation.

Other objects, advantages and features of the present
invention will become apparent to one of skill in the art
25   after review of the specification, claims and drawings.

Description of the Drawings

Fig. 1 graphs the dose-dependent impact of tocols on the
proliferation of melanoma B16 cells.

Figs. 2A and B illustrate the effects of combinations of
30   γ-tocotrienol, β-ionone and carvacrol on B16 melanoma cell
populations. Fig. 2A illustrates the effects of γ-tocotrienol
and β-ionone. Fig. 2B illustrates the effects of carvacrol
and β-ionone.

Fig. 3 is a survival curve for host mice receiving
35   isoprenoid-enriched diets following the detection of a solid
implanted B16 melanoma.

Description of the Invention

In one embodiment, the present invention is method of inhibiting the growth of tumor cells by exposing tumor cells to a combination of isoprenoids. In one embodiment of the present invention, the combination comprises at least one tocotrienol and at least one ionone. In another embodiment of the present invention, the combination comprises at least one tocotrienol and at least one statin. In a third embodiment of the present invention, the combination comprises at least one statin at least one ionone. We envision that another suitable embodiment of the present invention would be a combination comprising all three products of the mevalonate pathway.

By "tocotrienol," we mean a member of the following group: The vitamin E family consists of a mixture of vitamers, broadly consisting of tocopherols and tocotrienols. The tocotrienols are epimers of the corresponding tocopherols. The list below describes representative tocopherols and tocotrienols.

d-α-tocopherol	2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-chroman-6-ol
d-α-tocotrienol	2,5,7,8-tetramethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)-chroman-6-ol
d-β-tocopherol	2,5,8-trimethyl-2-(4,8,12-trimethyltridecyl)-chroman-6-ol
d-β-tocotrienol	2,5,8-trimethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)-chroman-6-ol
d-γ-tocopherol	2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)-chroman-6-ol
d-γ-tocotrienol	2,7,8-trimethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)-chroman-6-ol
d-δ-tocopherol	2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-chroman-6-ol
d-δ-tocotrienol	2,8-dimethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)-chroman-6-ol
d-tocopherol	2-methyl-2-(4,8,12-trimethyltridecyl)-chroman-6-ol
d-tocotrienol	2-methyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)-chroman-6-ol
2-desmethyltocotrienol	2-(4,8,12-trimethyltrideca-3,7,11-trienyl)-chroman-6-ol

Preferred tocotrienols include *d*-γ-tocotrienol and 2-desmethyltocotrienol. Other preferred tocotrienols include *d*-β-tocotrienol, *d*-δ-tocotrienol and *d*-tocotrienol.

-4-

By "ionone," we mean a member of the following group:
Ionones are carotenoid-related compounds widely distributed in
nature. α-Ionone and β-ionone and a number of oxygenated
derivatives are widely present in plants in free and conjugated

5   forms. Biological activities appear to be restricted to roles
as phytoalexins. Ionones are also formed by thermal and
photochemical mediated oxidation of carotenes.

Preferred ionones include β-ionone (4-2,6,6-trimethyl-1-
cyclohexen-1-yl)-3-buten-2-one; 6-10-dimethyl-undec-3,5-ene-

10   2,9-dione; 6,10-dimethyl-9,10-epoxy-undec-3,5-ene-2-one; 9,10-
diacetoxy-6,10-dimethyl-undec-3,5-ene-2-one; and 6,10-dimethyl-
9,10-diol-undec-3,5-ene-2-one. Other preferred ionones include
α-ionone (4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one.

By "statin," we mean a member of the following group: The

15   statins are derivatives of fungi metabolites (ML-
236B/compactin/monocalin K) isolated from *Pythium ultimum*,
Monacus ruber, *Penicillium citrinum*, *Penicillium brevicompactum*
and *Aspergillus terreus*. These analogs of 3-hydroxy-3-
methylglutaric acid (HMG), compete with HMG-CoA for the

20   substrate binding site on HMG-CoA reductase. Statins are
available by prescription in the U.S. For example, lovastatin
(Mevacor/Merck), simvastatin (Zocor/Merck), pravastatin
(Pravachol/Bristol-Myers Squibb) and fluvastatin
(Lescol/Sandoz). There are several more under clinical

25   investigation, including one in late-stage trials at Warner-
Lambert. The more lipophilic statins have been associated with
some skeletal muscle complaints (myositis, rhabdomyolysis), but
most of the side-effects reported in clinical trials have been
mild and tolerable (headache, abdominal pain, constipation,

30   flatulence and diarrhea). (Pedersen, T.R., N. Engl. J. Med.
333:1350-1351, 1995; Kobashigawa, J.A., et al., N. Engl. J.
Med. 333:621-627, 1995.) The following list discloses some
preferred statins.

Fungal derivatives	Trade name	Dosage range (mg/d)	Normal dose (mg/d)
lovastatin	Mevacor	10-80	20-40
pravastatin	Pravachol	10-40	20-40
simvastatin	Zocor	5-40	5-10
Synthetic compound			
fluvastatin	Lescol	20-80	20-40

-5-

The following list describes the chemical formula of preferred statins.

lovastatin: [1S[1a(R),3α,7β,8β(2S,4S),8aβ]]-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)ethyl]-1-maphthalenyl-2-methylbutanoate
pravastatin sodium: 1-Naphthalene-heptanoic acid, 1,2,6,7,8a-hexahydro-β,δ,6-trihydroxy-2-methyl-8-(2-methyl-1-oxybutoxy)-1-, monosodium salt [1S-[1α(βs,δS),2α,6α,8β(R),8aα
simvastatin: butanoic acid, 2,2-dimethyl-, 1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2 tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)ethyl]-1-napthalenyl ester [1S-[1α,3α,7β,8β,(2S,4S),-8aβ
sodium fluvastatin: [R,S-(E)]-(±)-7-[3(4-fluorophenyl)-1-(1-methylethyl)-1H-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid, monosodium salt

Preferred statins include lovastatin, pravastatin, simvastatin and fluvastatin.

The Examples below demonstrate a synergistic effect when B16 melanoma cells are treated with combinations of tocotrienols, lovastatin and ionone.

In one Example below, a 68% reduction in B16 melanoma cell number (measured after 48 hours) was obtained. This represented a synergistic effect of 9% over the additive sums of the individual effects. In *in vivo* tests measuring post-implant survival of melanoma-bearing mice, the P-values for the three comparisons of isoprenoid versus blend treatments fall into the range of 0.66 - 0.16 whereas the P-values for the three comparisons of isoprenoid versus isoprenoid treatments are greater than 0.88. We first note that all treatments significantly increased the duration of survival (P<0.03). Differences between single treatment effects were not significant. The nonparametric test P values fell between 0.64 and 0.95. When we tested the significance of differences between single treatment and blend effects the nonparametric P values fell between 0.16 and 0.64. The trend towards lower P values suggests a possible synergy.

By "synergistic," we mean a percentage reduction in cell number of at least an additional 5% over the additive sum of individual effects or an increase in host survivability of 5% over the additive sum of individual effects.

-6-

To reach the present invention, we evaluated the tumor-suppressive potency of a number of diverse isoprenoid compounds *in vitro* and the potency of two, d-γ-tocotrienol and β-ionone, *in vivo*. We build on findings that isoprenoids
5   bearing little commonality other than that of sharing a common precursor, isopentenyl pyrophosphate, suppressed melanoma cell proliferation and demonstrate that the effects of individual isoprenoids tested in binary mixtures are additive. We further report that a dietary-relevant intake of d-γ-
10   tocotrienol suppressed the growth of implanted tumors.

The study described below in the Examples estimated the concentrations of structurally diverse isoprenoids required to inhibit the increase in a population of murine B 16(F10) melanoma cells during a 48 hour incubation by 50% (IC_{50}
15   value). The IC_{50} values for d-limonene and perillyl alcohol, the monoterpenes in Phase I trials, were respectively 450 and 250 μmol/L; related cyclic monoterpenes (perillaldehyde, carvacrol, thymol), an acyclic monoterpene (geraniol) and the end ring analog of β-carotene (β-ionone) had IC_{50} values in
20   the range of 120-150 μmol/L. The IC_{50} value estimated for farnesol, the side chain analog of the tocotrienols (50 μmol/L) fell midway between that of α-tocotrienol (110 μmol/L) and those estimated γ-(20 μmol/L) and δ-tocotrienol (10 μmol/L). A novel tocotrienol lacking methyl groups on the
25   tocol ring proved to be extremely potent (IC_{50}, 0.9 μmol/L). In the first of two diet studies, experimental diets were fed to weanling C57BL female mice for 10 days prior to and for 28 days following the implantation of the aggressively growing and highly metastatic B16(F10) melanoma. The isomolar (116
30   μmol/kg diet) and the Vitamin E-equivalent (928 μmol/kg diet) substitution of d-γ-tocotrienol for dl-α-tocopherol in the AIN-76A diet produced 36% and 50% retardations respectively in tumor growth (P<0 05). In the second study, melanomas were established before mice were fed experimental diets formulated
35   with 2 mmol/kg d-γ-tocotrienol, β-ionone individually and in combination. A fourth diet was formulated with 4 mmol/kg d-γ-totrienol (Fig. 3). Each treatment increased (P<0.03) the duration of host survival. The present invention stems from

our finding that the effects of some individual isoprenoids
were additive and the effects of other individual isoprenoids
are synergistic. This finding suggests that particular
combination of isoprenoids would be uniquely suitable for
chemotherapeutic applications.

The methods of the present invention may be realized in
several embodiments. In one embodiment, the human or animal
subject is administered the isoprenoid combination in a
pharmaceutical or veterinary composition containing a safe and
effective dose. In another embodiment, the subject is fed a
food that has been enriched with the isoprenoid combination.
Statins are now available only by prescription and would not
preferably be added to food.

The human and animal foodstuffs and pharmaceutical
preparations for use in the methods of the present invention
are those containing the selected isoprenoid combination
additionally combined with conventional animal feeds, human
food supplements or approved pharmaceutical diluents and
excipients.

Highly active tocotrienols include d-γ-tocotrienol, d-δ-
tocotrienol and 2-desmethyltocotrienol, both of which occur
naturally. d-γ-tocotrienol and d-δ-tocotrienol may be
extracted from tocotrienol-rich fractions of rice brand oil or
of palm oil by published methods. 2-desmethyltocotrienol may
be extracted from tocotrienol-rich fractions of rice bran oil
by published methods.

β-Ionone may be obtained from a variety of commercial
sources. For example, β-ionone can be found at catalog no.
W25950-0, Aldrich Flavors and Fragrances; β-ionone is also
listed in Aldrich Fine Chemical and Sigma catalogs. Other
ionones are listed in the above catalogs and in Bedoukian
Research Distinctive Perfume & Flavor Ingredients.

Lovastatin may most easily be obtained commercially.

The isoprenoid combination, in addition to being added to
the subject's food, can be administered in the form of
pharmaceutical or veterinary compositions such as enteric
coated tablets, capsules, powders, solutions or emulsions.
The precise amount to be administered will depend upon the

-8-

isoprenoids employed, the route of administration, the weight
of the subject and the nature of the condition. We note that
the melanoma cell line used for the Examples below is known to
be extremely resistant to chemotherapeutic measures.
5   Therefore, the actual dosage used in patient treatment may be
less than that proposed from our experimental model.

Generally, the quantities of the agents required in a
pharmaceutical combination will provide a daily intake
substantially less than the quantities required when the
10   agents are administered individually. Perillyl alcohol, an
isoprenoid in clinical evaluation for efficacy as antitumor
agents tumor therapy, is adminstered at levels in excess of 8
g/day; lovastatin is clinically effective, but poorly
tolerated, for treating brain tumors at doses ~2 g/d (>20x the
15   dose administered for hypercholesterolemia). The tocotrienols
effectively lower cholesterol levels when consumed at dose
levels of <300 mg/day. Due to the synergistic actions of the
agents, we anticipate a preferable blend will contain 250 mg
tocotrienol/2000 mg ionone/150 mg statin (~2 mg/kg body wt.)
20   per day. A maximal preferred dosage would contain 500 mg
tocotrienol/4000 mg ionone/300 mg statin per day.

A representative pharmaceutical enteric coated tablet has
the formula:

Active principles:
25   d-γ-tocotrienol   50 mg
6,10-dimethyl-9,0-epoxy-undec-3,5-ene-2-one   400 mg
statin   30 mg
Excipients/fillers
Microcrystalline cellulose
30   Sodium starch glycolate
Corn starch
Hydrogenated vegetable oil wax
Magnesium stearate
Talc
35   The preferred normal per diem dosage is 5 tablets.

A bar or cookie may be of any desired type which is
prepared first and afterwards dosed with a small amount of
liquid containing the active principles:

-9-

d-γ-tocotrieno	150 mg
6,10-dimethyl-9,0-epoxy-undec-3,5-ene-2-one	400 mg

It is preferable that suitable flavorings are present in the bar or cookie to impart an acceptable level of

5   palatability. It is essential that the active principles are added after any baking or heating process has occurred and cooling is complete since the tocotrienols are heat labile. Such a bar or cookie may advantageously be packed in a hermetically sealed packet.

10 Because the tocotrienols and ionones are relatively non-toxic, doses larger than the higher figure stated above may be administered if desired, particularly with the enteric coated composition. Higher doses may, however, markedly reduce gut flora and lead to gastrointestinal disturbances. It is

15 recommended that when the compositions of the instant invention are administered, the daily intake of vitamin E is restricted.   The more lipophilic (fat loving) statins have been associated with some skeletal muscle complaints (myositis, rhabdomyolysis), but most of the side effects

20 reported in clinical trials have been mild and tolerable (headache, abdominal pain, constipation, flatulence and diarrhea. Patients (Thibault, et al., Clinical Cancer Research 2:483-491, 1996) tolerated intakes approaching 2 g/d (25 mg/kg body wt).

25 It will be apparent to those skilled in the art that numerous modifications or changes may be made without departing from the spirit or the scope of the present invention. Thus, the invention is only limited by the following claims.

30   EXAMPLES

Abbreviations used: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IC_{50}, the concentration required to suppress the increase in the population of melanoma cells by 50%; TRF, tocotrienol-rich fraction of palm oil; TRF_{25}, oryzanol-free

35 tocotrienol-rich fraction of rice bran oil.

A.   Materials and Methods

Isoprenoids: d-Limonene (97%), perillyl alcohol (99%),
perillaldehyde (92%), carvacrol (98%), thymol (98%), β-ionone
(96%), geraniol (98%), farnesol (96%) and dl-α-tocopherol

5   (97%) were purchased from Aldrich Chemical, Milwaukee, WI. An
abridged list of concentrated natural sources and aroma
characteristics of these isoprenoids appears below in Table 1.
A preliminary study revealed the very potent tumor-suppressive
action of the oryzanol-free tocotrienol-rich fraction of rice

10   bran oil (TRF$_{25}$) prepared by molecular distillation (Dr.
Laxman Singh, Vitamins, Inc., Chicago, IL). The fraction
consisted of 6% d-α-tocopherol, 12.5% d-α-tocotrienol, 21% d-
γ-tocotrienol, 10% d-δ-tocotrienol, 4.5% d-tocotrienol, 17% d-
2-desmethyl tocotrienol, 18% unidentified tocotrienol isomers

15   and 10% sterols and triglycerides (Qureshi, et al.,
unpublished data). The major constituents, d-α-tocotrienol,
d-γ-tocotrienol, d-δ-tocotrienol, and d-2-desmethyl
tocotrienol, were isolated by Advanced Medical Research,
Madison, WI. A chromatographic procedure was developed to

20   separate d-γ-tocotrienol from the tocotrienol-rich fraction
(TRF) of palm oil (36% d-γ-tocotrienol, 18% d-α-tocotrienol,
12% d-δ-tocotrienol and 22% d-α-tocopherol), a gift of the
Palm Oil Research Institute of Malaysia, Kuala Lumpur,
Malaysia. Silica gel (Merck, 60 μ, 150 g) suspended in hexane

25   was poured into a 350 mL glass funnel with a fritted disc.
The gel was washed with one L hexane prior to being loaded
with 5 g of the TRF in 20 mL hexane. The tocols were eluted
with the sequential applications of 500 mL mixtures of diethyl
ether (5%, 10%, 12%, 14%, 16%, 18%, 20%, 22%, 25% and 30%) in

30   hexane. The elution of each application of solvent into a
filter flask was speeded by the application of vacuum produced
by water aspiration. The eluates were taken to dryness under
vacuum, the residues redissolved in hexane and identified
according to retention time and absorption profile using an

35   analytical HPLC system. The fraction eluted with 18% diethyl
ether was predominantly (98%) d-γ-tocotrienol.

IC$_{50}$ Determinations: Murine B16(F10) melanoma cells, a
tumor cell line with high metastatic potential (Tsukamoto, et

-11-

al., 1991) obtained from Dr. William B. Ershler, were grown in
monolayer culture (35 x 10 mm flasks) in 3 mL RMPl 1640 media
(Sigma) supplemented with 10% newborn calf serum (GIBCOBRL,
Grand Island, NY) and 80 mg/L gentamycin (Sigma, St. Louis,

5   MO). Cultures, seeded with $1\text{-}1.5\text{x}10^5$ cells, were incubated
for 24 hours at 37°C in a humidified atmosphere of 5% CO_2.
Isoprenoids, dissolved in absolute ethanol, were added at 24
hours (0 time); all cultures contained 5 mL ethanol/L (85
μmol/L). The cultures were incubated for an additional 48

10 hours. The media was removed and the monolayers were washed
twice with Hanks' Balanced Salt Solution (Sigma) and then
incubated with a trypsin-EDTA solution (Sigma) at 37°C for 2
minutes. Trypsin was inactivated by suspending the cells in
medium containing 10% fetal bovine serum (Sigma). The cells

15 were pelleted at 250xg and resuspended in Hanks' Balanced Salt
Solution. Viable cells, cells that excluded 0.4% trypan blue
(GIBCOBRL), were counted with a hemocytometer; 24 hour cell
counts were deducted from final cell counts to provide an
estimate of the net increase in cell number. The calculation

20 of the concentration of an isoprenoid required to inhibit the
net increase in the 48 hour cell count by 50% (IC_{50}) is based
on plots of data from three or more evaluations.

        Animal Studies: This evaluation of the tumor-suppressive
potency of diverse isoprenoids was extended with two dietary

25 studies. The first examined the impact of a diet-relevant
intake of d-γ-tocotrienol on the growth of B16(F10) melanomas
in host mice. The second study evaluated the impact of
pharmacological intakes of d-γ-tocotrienol and β-ionone on the
post-implant survival of melanoma-bearing mice. We first

30 prepared a basal diet mix, patterned after the AIN-76A
formulation (American Institute of Nutrition, 1977) but free
of corn oil and dl-α-tocopherol. These ingredients and
Vitamin E-stripped corn oil were purchased from Teklad Test
Diets, Madison, WI. Stock solutions of dl-α-tocopherol (80

35 μmol/g) and d-γ-tocotrienol (80 μmol/g) were prepared with
vitamin E-stripped corn oil. Stock solutions of the tocols,
diluted with vitamin E-stripped corn oil, were mixed with the
basal diet to provide finished diets containing 5% corn oil

-12-

and specified tocol concentrations. Where noted, β-ionone was added to the oil. The diets, mixed weekly, were stored under refrigeration. Food cups were cleaned and refilled daily.

Experiment 1: Weanling C57BL female mice (Harlan-Sprague
5   Dawley, Madison, WI) were housed in groups of four on wood shavings in plastic cages and maintained at 25°C with a 12 hour light-dark cycle. The four groups of mice (20/group) were fed experimental diets for 10 days prior to and for 28 days following the implantation of B16 melanoma cells. The
10 split plot design consisted of four treatments comprised of two tocols, dl-α-tocopherol and d-γ-tocotrienol, each presented at two levels, 116 and 924 μmol/kg diet. This design permitted two comparisons of diets equal in tocol content and one comparison of diets about equal in d-α-
15 tocopherol equivalents. Lacking a definitive estimate of the biological activity of d-γ-tocotrienol, we considered reports that the 2, 5, 8 (d-β-) and 2, 7, 8 (d-γ-) trimethyl tocopherols have similar oxygen scavenging activity (maximally 66% that of the 2, 5, 7, 8 (d-α-) tetramethyl tocopherol) and
20 as a class, the tocotrienols have 5 - 30% of the biological activity of the tocopherols (Karnal-Eldin and Appelqvist, 1996) in developing the rough estimate that d-α-tocopherol has, minimally, 6-fold the biological activity of d-γ-tocotrienol. We then corrected for the biological activity of
25 the dl-α-tocopherol mixture (70% of the activity of d-α-tocopherol) in arriving at the 8:1 tocotrienol/tocopherol ratio used in formulating the experimental diets. Melanoma cells, cultured and harvested as previously, described (Shoff, et al., 1991), were washed twice with RPMI 1640 containing 10%
30 Fetal Bovine Sera (GIBCOBRL). The pelleted cells were suspended in RPMI 1640 and counted (98% viable) after a 1:20 dilution in 0.4% trypan blue. The cells were further diluted in RPMI 1640 (1 x 10^8 cells/L) and 0.1 mL of the suspension (1 x 10^4 cells) was injected subcutaneously into the flank of
35 each mouse. The study was terminated day 28 when the first mouse, a mouse in the control group, died. The mice were killed by CO_2 overdose and the tumors were excised and

weighed.   The protocol was reviewed and approved by College of
Agricultural and Life Sciences Animal Care Committee.

     Experiment 2:   Weanling female C57BL female mice (n=60,
Harlan Sprague-Dawley) were acclimated to the housing

5   conditions and AIN-76A diet (116 μmol dl-α-tocopherol/kg diet)
as described above.   Tumors were implanted and the mice
continued to receive the AIN-76A diet.   Beginning on day 8
post-implant the mice were palpated daily for the presence of
a tumor.   Tumors were first detected on day 14.   Random

10 numbers were generated for assigning each mouse in a
sequential subset of five to a diet.   Experimental treatments
provided 2 and 4 mmol d-γ-tocotrienol/kg diet.   We
additionally tested the impact of β-ionone (2 mmol β-ionone/kg
AIN-76A diet) and that of a blend of the two isoprenoids (2

15 mmol each/kg diet) on the survival of the mice.   The mice were
continued on the respective experimental diets; moribund mice,
identified by the Research Animal Resource-trained supervisor
blinded to the experimental design, were killed by CO_2
overdose and the tumors were excised and weighed.   The

20 protocol was reviewed and approved by College of Agricultural
and Life Sciences Animal Care Committee.

     Statistical Methods: StatView and SuperANOVA software
(Abacus Concepts, Berkeley, CA) were used for the assessment
of treatment-mediated effects.   Treatment-mediated differences

25 in body and tumor weights, days to tumor appearance and days
to morbidity were identified with split plot analysis of
variance and pairwise t-tests of least squares means.
Treatment-mediated differences in days to morbidity were also
assessed using parametric (paired t-test) and nonparametric

30 (Wilcoxon signed rank) tests (Haycock, et al., 1992).

B.   Results

     Fig. 1 is a representative evaluation of the dose-
dependent impact of five different tocols on the proliferation
of melanoma B16 cells.   Cultures (3 mL) seeded with 1-1.5 x

35 10^5 cells were incubated for 24 hours prior to the
introduction of the tocols.   Viable cells were counted at 48
hours following the addition of the tocols.   The cell count at

-14-

24 hours (0 time) is shown by the dashed line. The intersection of the solid horizontal line and the line plotted for each tocol indicates the concentration at which the tocol suppressed by 50% the increase in cell number during the incubation (IC_{50} value). IC_{50} values (mean, SD and n) for all isoprenoids tested are listed on Table 1. Referring to Fig. 1, dl-α-Tocopherol had no impact on cell number. The growth-suppressive potency of the individual tocotrienols was inverse to the number of methyl groups on the 6-chromanol ring: d-α-tocotrienol (methyl groups at carbons 5, 7, 8 and 2) << d-γ-tocotrienol (methyl groups at carbons 7, 8 and 2) < d-δ-tocotrienol (methyl groups at carbons 8 and 2) << d-2-desmethyl tocotrienol (no methyl groups) (Table 1).

Within this series of tocotrienols the IC_{50} value was inversely related to the number of methyl groups on the 6-chromanol ring. Similarly, the IC_{50} values calculated from plots for the more polar monoterpenoid alcohols, perillyl alcohol ([R]4-isopropenyl- 1-methanol-cyclohexene), perillaldehyde ([R]-4-isopropenyl-1-carboxaldehyde cyclohexene), thymol (5-methyl-2-isopropylphenol), carvacrol (5-isopropyl-2-methylphenol) and geraniol (trans 3,7-dimethyl-2,6-octadien-1-ol) were much lower than that for d-limonene ([R]4-isopropenyl-1-methyl-1-cyclohexene). The IC_{50} value for β-ionone, the end ring analog of β-carotene, matched those of the more polar monoterpenes (Table 1). The IC_{50} for farnesol (trans, trans 3,7,11-trimethyl-2,6,10 dodecatrien-1-ol), a sesquiterpene and a structural analog to the side chain of the tocotrienols, fell midway between those of d-α-tocotrienol and d-γ- and d-δ-tocotrienol.

The first dietary study evaluated the impact of d-γ-tocotrienol and dl-α-tocopherol on days to detection of a solid tumor and growth of implanted B16 melanomas. As noted above the design permitted two comparisons of treatments providing equal tocol concentration (116 and 928 μmol/kg diet) and one comparison of treatments providing about 80 μmol d-α-tocopherol equivalents (35 mg)/kg diet. At the time of tumor implant the body weight of mice receiving the high tocopherol diet was significantly lower than that of mice receiving the

-15-

low tocol diets (Table 2). At 28 days post-implant the melanomas accounted for 15% of the weight of mice receiving the AIN-76A diet. Two tocols were tested, each at two levels. The split plot analysis of variance showed the effects of

5   tocols (P<0.001) and levels (P<0.04) on 28 day tumor weight were significant; there was no evidence of an interaction between the two factors (P<0.77). Least mean squares analyses confirmed the tumor growth-suppressive action of γ-tocotrienol (Table 2). The other measure of tumor growth, days post-

10   implant to tumor detection showed tocol (P<0.03), level (P<0.01) and the interaction (P<0.02) to be significant. The least mean squares analysis showed that the effect of the treatment providing 928 μmol d-γ-tocotrienol/kg diet on this measure of tumor growth differed significantly from the

15   effects of the other treatments.

       We next determined that the effects of the isoprenoids on the growth of B16 melanoma cells in culture are additive, and in some cases, synergistic. Fig. 2A illustrates the additive effects of γ-tocotrienol and β-ionone on B16 melanoma cell

20   populations. Values are means ± SD, n=28; pooled SEM = 36 (x10^4). Cell counts calculated to show the decreased population relative to the control are listed on the insert table.

       Fig. 2B illustrates the additive effects of carvacrol and

25   β-ionone on B16 melanoma cell populations. Values are means ± SD, n=28; pooled SEM = 62 (x10^4). Cell counts calculated to show the decreased population relative to the control are listed in the inset table.

       [a-h] Means not sharing a superscript (a>b>c>d>e>f>g>h) are

30   different (P<0.001). Referring to Fig. 2, B16 melanoma cells were incubated with β-ionone and d-γ-tocotrienol in one test (Fig. 2A). d-γ-Tocotrienol (7.5 μmol/L) and β-ionone (50 μmol/L) reduced the 48 hour cell count by 19% and 10% respectively (Fig. 2A). The 34% reduction in cell number

35   achieved with the paired isoprenoids exceeded the 29% reduction predicted by the sum of the individual effects (Fig. 2A, inset). At higher concentrations d-γ-tocotrienol (15 μmol/L) and β-ionone (100 μmol/L) reduced the 48 hour cell

count by 34% and 16% respectively; the additive effect, a 68%
reduction in cell number, was also greater than the sum of the
individual effects (59%) (Fig. 2A, inset).

Whereas the foregoing results pointed to a synergistic
5   action of d-γ-tocotrienol and β-ionone, the study pairing
carvacrol and β-ionone revealed only an additive effect (Fig.
2B). Carvacrol (50 μmol/L) and β-ionone (75 μmol/L) reduced
the 48 hour cell count by 25% and 31% respectively; the
additive effect, a 48% reduction in cell number, was less than
10 the 56% reduction predicted by the sum of the individual
effects. Carvacrol (100 μmol/L) and β-ionone (150 μmol/L)
reduced the cell count by 35% and 65% respectively; the
additive effect, an 84% reduction in cell number, again was
less than the 100% reduction predicted by the sum the
15 individual effects (Fig. 2B, inset). The reduction in cell
number predicted by the sum of the individual effects of the
isoprenoids was highly correlated with that achieved with the
paired isoprenoids (r=0.91, n=8, P<0.01).

We then asked if either dietary d-γ-tocotrienol or β-
20 ionone would prolong the survival of mice bearing implanted
melanomas. Dietary treatments were initiated following the
detection of solid tumors. We also asked whether these
structurally diverse isoprenoids would have an additive effect
on survival. The experimental groups were constructed by
25 random assignment of each member of successive subsets of 5
mice to one of the experimental diets. Time to tumor
detection did not vary between groups (Table 3). Treatments
increased median duration of survival by 42 ± 4.5% and the
mean duration of survival by 30% (P<0.005); differences
30 between duration of survival means of the experimental groups
were not significant (Table 3, Fig. 3). Fig. 3 is a survival
curve for host mice receiving isoprenoid-enriched diets
following the detection of a solid implanted B16 melanoma.
Mean survival durations are presented on Table 4. β-ionone
35 and γ-tocotrienol[1] treatments provided 2 mmol of the
isoprenoid/kg diet; the blend provided 2 mmol of each
isoprenoid/kg diet; and γ-tocotrienol[2] provided 4 mmol γ-
tocotrienol/kg diet.

-17-

According to the experimental design, each animal within a subset of 5 could be paired with another member of the subset for testing. P-values for the pairwise comparisons, assuming normal and anormal distributions, are listed on Table
5   4. All analyses revealed highly significant differences between the control and treatment group means; differences between the treatment group means were not significant. Lending additional creditability to the results of the *in vitro* analysis showing an at least additive effect of the two
10   isoprenoids is the trend shown on Table 4. Whereas the P-values for the three comparisons of isoprenoid versus isoprenoid treatments are greater than 0.88, the P-values for the three comparisons of isoprenoid versus blend treatments fall in the range 0.66 - 0.16.

15   C.   Discussion

The B16(F10) melanoma provides a rigorous model for assessing, *in vitro* and *in vivo*, the potency of pharmacological agents (Gruber, et al., 1992; Kuwashima, et al., 1990; Mac Neil, et al., 1992; Tsukamoto, et al., 1991;
20   Shoff, et al., 1991). We now report that a diet providing an intake of 0.4 μmol d-γ-tocotrienol/d suppressed the growth of the B16 melanoma implanted in the flank of host mice. In our more rigorous test, a diet providing 7 μmol d-γ-tocotrienol or β-ionone/d suppressed the growth of established B16 melanomas.
25   *In vitro* tests provided evidence of the additive effects of these two isoprenoids (Fig. 2A, B). The diet providing an intake of 7 μmol d-γ-tocotrienol/d increased the duration of survival by 35%. Whereas doubling the intake of d-γ-tocotrienol to 14 μmol/d did not further increase the duration
30   of survival (-0.21 d, P=0.95), adding an intake of 7 μmol of β-ionone to that of 7 μmol d-γ-tocotrienol/d tended to increase the duration of survival (+0.83 d, P=0.65, Tables 3, 4). This study evaluated the responses of established implanted melanomas to β-ionone and d-γ-tocotrienol, two
35   isoprenoids with structural relationships to the two antioxidant nutrients recently discounted as having tumor-suppressive actions (Greenberg and Sporn, 1996). Both

isoprenoids significantly increased survival time (Table 4).
We note findings that a massive intake of ascorbic acid (733
μmol/d) suppressed the growth of implanted B16 melanomas
(Meadows, et al., 1991). Our studies showed that a diet

5   providing an 8-fold elevation in d-α-tocopherol equivalents
had a marginal impact on tumor growth whereas a d-γ-
tocotrienol diet providing only the d-α-tocopherol-equivalent
of the AIN-76A diet yielded a highly significant suppression
of tumor growth.

10   D.   Synergistic Action of Lovastatin, Tocotrienol and Ionone
i. Background
Lovastatin, a fungal antibiotic, competitively inhibits
3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase
activity (J.L. Goldstein and M.S. Brown, Nature 343:425-430,

15   1990). β-Ionone (S.G. Yu, et al., J. Agric. Food Chem.
42:1493-1496, 1994) a pure isoprenoid, and related agents
suppress HMG CoA reductase activity via the suppression of its
synthesis (reviewed in H. Mo, et al., Nutritional Oncology, D.
Huber & G. Blackburn, eds. Academic Press, NY, in press; C.E.

20   Elson, et al., Amer. Assoc. Cancer Res., in press). The
tocotrienols, a group of mixed isoprenoids, suppress reductase
activity by triggering the proteolytic degradation of the
enzyme (C.E. Elson, supra, in press). As a consequence of the
inhibited reductase action, lovastatin and later derivatives

25   (J.L. Goldstein and M.S. Brown, supra, 1990; R.A. Parker, et
al., J. Biol. Chem. 268:11230-11238, 1993), β-ionone and
related pure isoprenoids (S.G. Yu, et al., supra, 1994; H. Mo,
et al., supra, in press), and the tocotrienols (H. Mo, et al.,
supra, in press; R.A. Parker, et al., supra, 1993; A.A.

30   Qureshi, et al., J. Biol. Chem. 261:10544-10550, 1986; A.A.
Qureshi, et al., Proceedings of the 1996 PORIM International
Palm Oil Conference, Kuala Lumpur, Malaysia, pp. 168-180,
1996) lower serum cholesterol levels. Cells treated with
lovastatin have a several-fold increase in HMG CoA reductase,

35   an increase resulting in an elevated reductase activity
following the removal of the inhibitor (J.L. Goldstein and
M.S. Brown, supra, 1990). Cellular activities underlying the

-19-

increase in reductase mass include increases in the
transcription and efficiency of processing of HMG CoA
reductase mRNA and a decrease in the proteolytic degradation
of the enzyme. β-Ionone (S.G. Yu, et al., supra, 1994) and
5   the tocotrienols (R.A. Parker, et al., supra, 1993) attenuate
the degree of elevation in reductase activity (S.G. Yu, et
al., supra, 1994) and mass (R.A. Parker, et al., supra, 1993)
effected by lovastatin treatment.

          Mevalonate pathway intermediates are essential for the
10   post-translational modification of proteins that play
essential roles in cell proliferation (see H. Mo, et al.,
supra, in press). The competitive inhibition of mevalonate
synthesis imposed by the statins suppresses the proliferation
of cultured cells (J.L. Goldstein and M.S. Brown, supra, 1990)
15   and the growth of implanted tumors (W. Maltese, et al., J.
Clin. Invest. 76:1748-1754, 1985; J.P. Jani, et al., Invasion
Metastasis 13:314-324, 1993). A five-year follow-up of 745
hypercholesterolemic patients receiving lovastatin, up to 80
mg/d, revealed a 33% reduction in cancer incidence (14
20   observed, 21 predicted) (J.A. Tolbert, Arch. Intern. Med. 153-
1079-1087, 1993). A Phase I trial evaluated the tolerability
of lovastatin administered at progressively higher doses (3 to
43 cycles of 7-day courses given monthly) involving 88
patients with solid tumors (39 with brain tumors, 24 with
25   hormone-independent prostate cancer). Doses ranged between 2
and 45 mg/kg (the maximum dose for hypercholesterolemia is 80
mg/d). Sixty patients experienced a total of 128 episodes of
toxicity; the incidence and severity of toxicity increased
markedly once the 25 mg/kg dose level was reached. To prevent
30   myotoxicity and improve the tolerability of lovastain,
ubiquinone was administered to a cohort of patients receiving
30 mg/kg or higher doses of lovastatin. Ubiquinone did not
decrease the incidence of musculoskeletal toxicity but
significantly decreased its severity. The authors conclude
35   with the suggestion that high-grade gliomas represent a
reasonable target for Phase II trials and recommended that
alternative treatment schedules aimed at achieving sustained

inhibition of mevalonate synthesis be investigated (A.
Thibault, et al., Clin. Cancer Res. 2:483-491, 1996).

   ii.   Ionone, Tocotrienol and Lovastatin Act
         Synergistically to Inhibit Tumor Cell Growth

5   β-Ionone, γ-tocotrienol and lovastatin are known to
individually suppress the proliferation of murine melanoma
B16/F10 cells. IC_{50} values for β-ionone and γ-tocotrienol are
140 μmol (L. He, et al., J. Nutr. 127:668-674, 1997), and 20
μmol/l (L. He, et al., supra). Using the protocol outlined in
10 He, et al., supra, we have determined that B16 cells incubated
with 1.9 ± 0.3 μmol/l lovastatin grow at 61.4 ± 4.6% the rate
of controls. This value confirms the value determined using a
colony formation assay (J.P. Jani, et al., supra, 1993). In
vivo studies show that lovastatin (50 mg/kg) administered ip
15 on alternate days (J.L. Goldstein and M.S. Brown, supra,
1990), β-ionone (2 mmol/kg diet) (L. He, et al., supra, 1997)
and γ-tocotrienol (116-2000 μmol/kg diet) individually
suppressed the growth implanted murine melanoma B16F10 tumors.
        Our in vitro assays record synergy between the growth
20 suppressive actions of the three agents. The growth-
suppressive action of each combination is greater than that
predicted by the sum of the individual effects (Table 5).
        Table 5 tabulates the results of our in vitro assays.
Table 5 lists various concentrations of lovastatin, β-ionone
25 and γ-tocotrienol and the growth suppressive ability of each
treatment. Growth in treated cells is recorded as % of
control growth. On Table 5 we show that 2 μM lovastatin and
150 μM β-ionone respectively decreased cell growth by 50% and
32% (50% and 68% of control growth)--the combined action of
30 the two compounds is predicted to suppress growth by 82% (50%
+ 32%) or reduce cell growth to 18% of control. Growth was
reduced by 90% or to 10% of control. In a series of tests
combining lovastatin with an isoprene (tocotrienol or ionone)
we predicted a 66% reduction in cell count (33±5% of control).
35 However, we observed an 87% decrease (13±4% of control).
Combinations of isoprenoids yielded a 50% reduction in cell
growth--the predicted reduction was 57 (50% of control
predicted, 43% of control obtained).

One will note that in each combination of lovastatin, β-ionone and tocotrienol, the combination resulted in less cell growth than the predicted, additive value.

Referring to Table 5, we would predict a full suppression
5   of growth with a blend providing 2 μM lovastatin, 5 μM tocotrienol and 50 μM ionone.

-22-

APPENDIX 1 - TABLES

Table 1. The concentrations of selected isoprenoids required to suppress the increase in the population of melanoma cells by 50% during a 48 hour incubation[1]. Also listed are representative sources and aroma characteristics of each isoprenoid.

Isoprenoid Class	n μmol/L	IC_{50}	Representative Source	Aroma
Monoterpenes				
d-Limonene	3	450±43	Citrus Peel, Mint	lemon
Perillyl alcohol	3	250±28	Citrus Peel, Mint, Sage, Lavender	lilac
Geraniol	3	150±19	Citrus Peel, Basil, Rosemary	fruit
Perillaldehyde	3	120±17	Citrus, Basil, Rosemary	fruit
Carvacrol	3	120±15	Thyme, Marjoram, Mint, Dill	mint
Thymol	3	120±15	Thyme, Oregano, Tangerine Peel	thyme
Sesquiterpenes				
Farnesol	2	50	Rose, Chamomile, Lavender, Lilac	lilac
β-Ionone	5	140±23	Grapes, Corn, Apricots, Prunes	woody
Tocols				
d-α-Tocotrienol	4	110±15	Barley, Rice, Oat Palm, Olive Oils	oily
d-γ-Tocotrienol	6	20±3	Barley, Rice, Oat Palm, Olive Oils	oily
d-δ-Tocotrienol	3	10±3	Barley, Rice, Oat Palm, Olive Oils	oily
d-2-desmethyl Tocotrienol	3	0.9±0.2	Rice Bran	oily
dl-α-Tocopherol	4	>1600	Soybean, Corn, Wheat Barley, Rice, Oats, Palm Oils	oily

[1] Values are means ± SD.

Table 2. Impact of *d*-γ-tocotrienol on days to detection and 28-d growth of B16 melanomas implanted into the flanks of mice[1].

Tocol	μmol/kg	body weight		tumor		liver
	diet	10 d	38 d	detection	weight	weight
	(g)	(g)		(d)	(g)	(g)
dl-α-Tocopherol	116	17.33ᵃ	23.30	19.55ᵇ	3.59ᵃ	1.18ᵃ
d-γ-Tocotrienol	116	17.02ᵃ	22.43	19.68ᵇ	2.31ᵇᶜ	1.12ᵃᵇ
dl-α-Tocopherol	928	16.41ᵇ	22.53	19.75ᵇ	2.89ᵃᵇ	1.06ᵇ
d-γ-Tocotrienol	928	16.83ᵃᵇ	22.56	22.90ᵃ	1.78ᶜ	1.03ᵇ
pooled SEM		0.10	0.19	0.35	0.17	0.02
	a>b		a>b	a>b>c	a>b	

a-c Means not sharing a superscript are different (P<0.05).
[1] Values are means, n=20.
[2] Days post implant for appearance of a palpable tumor.

Table 3. Impact of isoprenoid-enriched diets on the duration of survival of host mice bearing established melanomas[1].

-23-

Group	d-γ-Tocotrienol	β-Ionone	Days to	Days survival	
	mmol/kg diet		tumor	Mean	Median
Control	0	0	15.00	13.83[b]	15.0
d-γ-Tocotrienol	2	0	14.92	18.67[a]	20.0
d-γ-Tocotrienol	4	0	14.92	18.46[a]	22.0
β–Ionone	0	2	14.92	18.27[a]	23.0
Blend	2	2	14.92	19.50[a]	20.5
pooled SEM				0.547 a>b	

[a-b] Means not sharing a superscript are different (P<0.03).
[1] Values are means, n=12.

Table 4. Pairwise comparisons of isoprenoid effects on the duration of survival following detection of a B16 melanoma in the flanks of mice.

	Paired t-test		Wilcoxon Signed Rank	
Group Comparison	t-value	P-value	Z-value	P-value
Control vs.				
d-γ-Tocotrienol[1]	5.74	0.01	-2.93	0.01
d-γ-Tocotrienol[2]	2.70	0.02	-2.22	0.03
β-Ionone[3]	2.81	0.02	-2.18	0.03
Blend[4]	3.27	0.01	-2.45	0.01
d-γ-Tocotrienol[1] vs.				
d-γ-Tocotrienol[2]	0.46	0.66	-0.47	0.64
β-Ionone[3]	0.06	0.95	-0.15	0.88
Blend[4]	0.46	0.66	-0.47	0.64
d-γ-Tocotrienol[2]				
β-Ionone[3]	0.11	0.92	-0.06	0.95
Blend[4]	1.36	0.21	-1.42	0.16
β-Ionone[3] vs.				
Blend[4]	0.93	0.37	-0.83	0.41

[1] 2 mmol *d*-γ-tocotrienol/kg diet.
[2] 4 mmol *d*-γ-tocotrienol/kg diet.
[3] 2 mmol β-ionone/kg diet.
[4] 2 mmol *d*-γ-tocotrienol + 2 mmol β-ionone/kg diet.

Table 5

Lovastatin	β-Ionone	γ-Tocotrienol	Growth	Predicted
	μmol/L		% of Control	
2	0	0	50	
0	150	0	68	
2	150	0	10	18
2	0	0	60	
0	0	5	90	
0	0	10	85	
2	0	5	32	50
2	0	10	20	45
1.5	0	0	71	
3	0	0	53	
0	0	10	72	
0	0	20	65	
1.5	0	10	20	43
1.5	0	20	10	36
3	0	10	0	25
3	0	20	0	18
0	50	0	90	
0	100	0	84	
0	0	7.5	81	
0	0	15	66	
0	50	7.5	66	71
0	100	7.5	64	65
0	50	15	51	56
0	100	15	32	50
0	150	0	32	
0	0	15	77	
0	150	15	0	9

-25-

APPENDIX 2 - LITERATURE CITED

Adany, I., et al., "Differences in sensitivity to farnesol toxicity between neoplastically- and non-neoplastically-derived cells in culture," Cancer Lett. 79:175-179, 1994.

American Institute of Nutrition. Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J. Nutr. 107:1340-1348, 1977.

Block, G.,et al., "Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence," Nutr. Cancer 18:1-29, 1992.

Coni, P., et al., "Hypomethylation of β-hydroxy-β-methylglutaryl coenzyme A reductase gene and its expression during hepatocarcinogenesis in the rat," Carcinogenesis 13:497-499, 1992.

Correll, C.C., et al., "Identification of farnesol as the non-sterol derivative of mevalonic acid required for the accelerated degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase," J. Biol. Chem. 269:17390-17393, 1994.

Counts, J.L. and Goodman, J.I., "Hypomethylation of DNA: a possible epigenetic mechanism involved in tumor promotion," Prog. Clin. Biol. Res. 391:81-101, 1995.

Crane, D., "Biosynthesis of sesquiterpenes. In: Biosynthesis of Isoprenoid Compounds (Porter, J.W. and Spurgeon, S.L., eds.) vol. 1, pp. 283-374. John Wiley & Sons, New York, NY, 1981.

Croteau, R., "Biosynthesis of monoterpenes. In: Biosynthesis of Isoprenoid Compounds (Porter, J.W. and Spurgeon, S.L., eds.) vol. 1, pp. 225-282. John Wiley & Sons, New York, NY, 1981.

Crowell, P.L., et al., "Selective inhibition of isoprenylation of 21-26-kDa proteins by the anticarcinogen d-limonene and its metabolites," J. Biol. Chem. 266:17679-17685, 1991.

Cuthbert, J.A. and Lipsky, P.E., "Suppression of the proliferation of ras-transformed cells by fluoromevalonate, an inhibitor of mevalonate metabolism," Cancer Res. 55:1732-1740, 1995.

DeClue, J.E., et al., "Inhibition of cell growth by lovastatin is independent of ras function," Cancer Res. 51:712-717, 1991.

Elson, C.E., "Suppression of mevalonate pathway activities by dietary isoprenoids: Protective roles in cancer and cardiovascular disease," J. Nutr. 125:1666s- 1672s, 1995.

Elson, C.E. and Qureshi, A.A., "Coupling the cholesterol- and tumor-suppressive actions of palm oil to the impact of its minor constituents on 3-hydroxy-3-methylglutaryl coenzyme A

reductase activity," <u>Prost. Leuko. Essen. Fatty Acid</u> 52:205-208, 1995.

Elson, C.E. and Yu, S.G., "The chemoprevention of cancer by mevalonate-derived constituents of fruits and vegetables," <u>J. Nutr.</u> 124:607-614, 1994.

Gould, M.N., "Prevention and therapy of mammary cancer by monoterpenes," <u>J. Cell. Biochem.</u> S22:139-144, 1995.

Gould, M.N.,<u>et al.</u>, "A comparison of tocopherol and tocotrienol for the chemoprevention of chemically-induced mammary tumors," <u>Am. J. Clin. Nutr.</u> 53:1068S-1070S, 1991.

Greenberg, E.R. and Sporn, M.B., "Antioxidant vitamins, cancer and cardiovascular disease," <u>New Engl. J. Med.</u> 334:1189-1190, 1996.

Gruber, J.R., <u>et al.</u>, "Increased expression of protein kinase Cα plays a key role retinoic acid-induced melanoma differentiation," <u>J. Biol. Chem.</u> 267:13356-13360, 1992.

Haycock, K.A., <u>et al.</u>, "Nonparametrics. In: StatView, pp. 344-355. Abaacus Concepts, Berkeley, CA, 1992.

Kamal-Eldin, A. and Appelqvist, L-A., "The chemistry and antioxidant properties of tocopherols and tocotrienols," <u>Lipids</u> 31:671-701, 1996.

Kawata, S., <u>et al.</u>, "Increase in the active form of 3-hydroxy-3-methylglutaryl coenzyme A reductase in human hepatocellular carcinoma: Possible mechanism for alteration of cholesterol biosynthesis," <u>Cancer Res.</u> 50:3270-3273, 1990.

Kohl, N.E., <u>et al.</u>, "Development of inhibitors of protein farnesylation as potential chemotherapeutic agents," <u>J. Cell. Biochem.</u> 22:145-150, 1995.

Komiyama, K., <u>et al.</u>, "Studies on the biological activity of tocotrienols," <u>Chem. Pharm. Bull.</u> 37:1369-1371, 1989.

Kuwashima, Y., <u>et al.</u>, "Responses of a murine B16 melanoma to pharmacotherapy studied and compared with different assay systems," <u>Cancer Res. Clin. Oncol.</u> 116:173-178, 1990.

Laird, P.W. and Jaenisch, R., "DNA methylation and cancer," <u>Human Molec. Gen.</u> 3:1487- 1495, 1994.

Mac Neil, S., <u>et al.</u>, "Signal transduction in murine B16 melanoma cells," <u>Melanoma Res.</u> 2:197-206, 1992.

Meadows, G.G., <u>et al.</u>, "Ascorbate in the treatment of experimental transplanted melanoma," <u>Am. J. Clin. Nutr.</u> 54:1284s- 1291s, 1991.

-27-

Meigs, T.E., _et al._, "Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A degradation by the nonsterol mevalonate farnesol _in vivo_," _J. Biol. Chem._ 271:7916-7922.

Parker, R.A., _et al._, "Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase," _J. Biol. Chem._ 268:11230-11238, 1993.

Perez-Sala, D. and Mollinedo, F., "Inhibition of isoprenoid biosynthesis induces apoptosis in human promylocytic HL-60 cells," _Biochem. Biopys. Res. Commun._ 199:1209-1215, 1994.

Qureshi, A.A., _et al._, "Response of hypercholesterolemic subjects to administration of tocotrienols," _Lipids_ 30:1171-1177, 1995.

Rossiello, M.R., _et al._, "Similar patterns of hypomethylation in the β-hydroxy-β-methylglutaryl coenzyme A reductase gene in hepatic nodules induced by different carcinogens," _Molec. Carcinogenesis_ 10:237-245, 1994.

Shoff, S.M., _et al._, "Concentration-dependent increase in murine P388 and B16 population doubling time by the acyclic monoterpene geraniol," _Cancer Res._ 51:37-42, 1991.

Thibault, A., _et al._, "Phase I study of phenylacetate administration twice daily to patients with cancer," _Cancer_ 75:2932-2938, 1995.

Thibault, A., _et al._, "Phase I studies of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer," _Clin. Cancer Res._ 2:483-491, 1996.

Tsukamoto, K., _et al._, "Malignant melanoma: relationship to parameters of differentiation," _Melanoma Res._ 1:223-230, 1991.

Vasudevan, S., _et al._, "Hypomethylation of β-hydroxy-β-methylglutaryl coenzyme A (HMG CoA) reductase gene in polyps and cancers of human colon," _FASEB J._ 8:A647, 1994 (abs.).

West, C., "Biosynthesis of diterpenes. In: Biosynthesis of Isoprenoid Compounds (Porter, J.W. and Spurgeon, S.L., eds.) vol. 1, pp. 375-411, John Wiley & Sons, New York, NY, 1981.

Yu, S.G., _et al._, "Geraniol, an inhibitor of mevalonate biosynthesis, suppresses the growth of hepatomas and melanomas transplanted to rats and mice, _J. Nutr._ 125:2763-2767, 1995.

-28-

CLAIMS

I claim:

1.   A method of inhibiting the growth of tumor cells comprising the step of exposing tumor cells to an effective amount of a composition comprising at least two compounds selected from the group consisting of tocotrienols, statins, 5 and ionones, wherein the amount is effective to inhibit tumor cell growth.

2.   The method of claim 1 wherein the composition comprises a tocotrienol and an ionone.

3.   The method of claim 1 wherein the composition comprises a tocotrienol and a statin.

4.   The method of claim 1 wherein the composition comprises a statin and an ionone.

5.   The method of claim 1 wherein the composition comprises a statin, an ionone and a tocotrienol.

6.   The composition of claim 1 wherein the tocotrienol is selected from the group consisting of d-γ-tocotrienol, 2-desmethyltocotrienol, d-δ-tocotrienol and d-tocotrienol.

7.   The method of claim 1 wherein the tocotrienol is d-γ-tocotrienol.

8.   The method of claim 1 wherein the ionone is selected from the group consisting of β-ionone and α-ionone.

9.   The method of claim 1 wherein the ionone is β-ionone.

10.   The method of claim 1 wherein the statin is lovastatin.

SUBSTITUTE SHEET (RULE 26)

11. The method of claim 1 wherein the exposing step comprises administering the composition in the form of a food.

12. The method of claim 1 wherein the exposing step comprises administering the composition in the form of a pharmaceutical.

13. A method of inhibiting tumor growth comprising the step of exposing a patient to an effective amount of a composition comprising at least two compounds selected from the group consisting of tocotrienols, statins and ionones, 5 wherein the amount is effective to inhibit tumor growth.

14. A pharmaceutical composition comprising an effective amount of at least two compounds selected from the group consisting of tocotrienols, statins and ionones wherein the amount is effective to inhibit tumor growth.

15. The pharmaceutical composition of claim 14 comprising at least one tocotrienol and at least one ionone.

16. The pharmaceutical composition of claim 14 comprising at least one tocotrienol and at least one statin.

17. The pharmaceutical composition of claim 14 comprising at least one statin and at least one ionone.

18. The pharmaceutical composition of claim 14 comprising at least one statin, at least one ionone and at least one tocotrienol.

19. The pharmaceutical composition of claim 14 comprising β-ionone and d-γ-tocotrienol.

20. The composition of claim 14 wherein the composition is part of a food.

SUBSTITUTE SHEET (RULE 26)

21.   The composition of claim 14 wherein the daily intake
of tocotrienol is between 250 mg and 500 mg.

22.   The composition of claim 14 wherein the daily intake
of ionone is between 2000 mg and 4000 mg.

23.   The composition of claim 14 wherein the daily intake
of statin is between 150 mg and 300 mg.

24.   The composition of claim 18 wherein the daily intake
of tocotrienol is between 250 mg and 500 mg, of ionone is
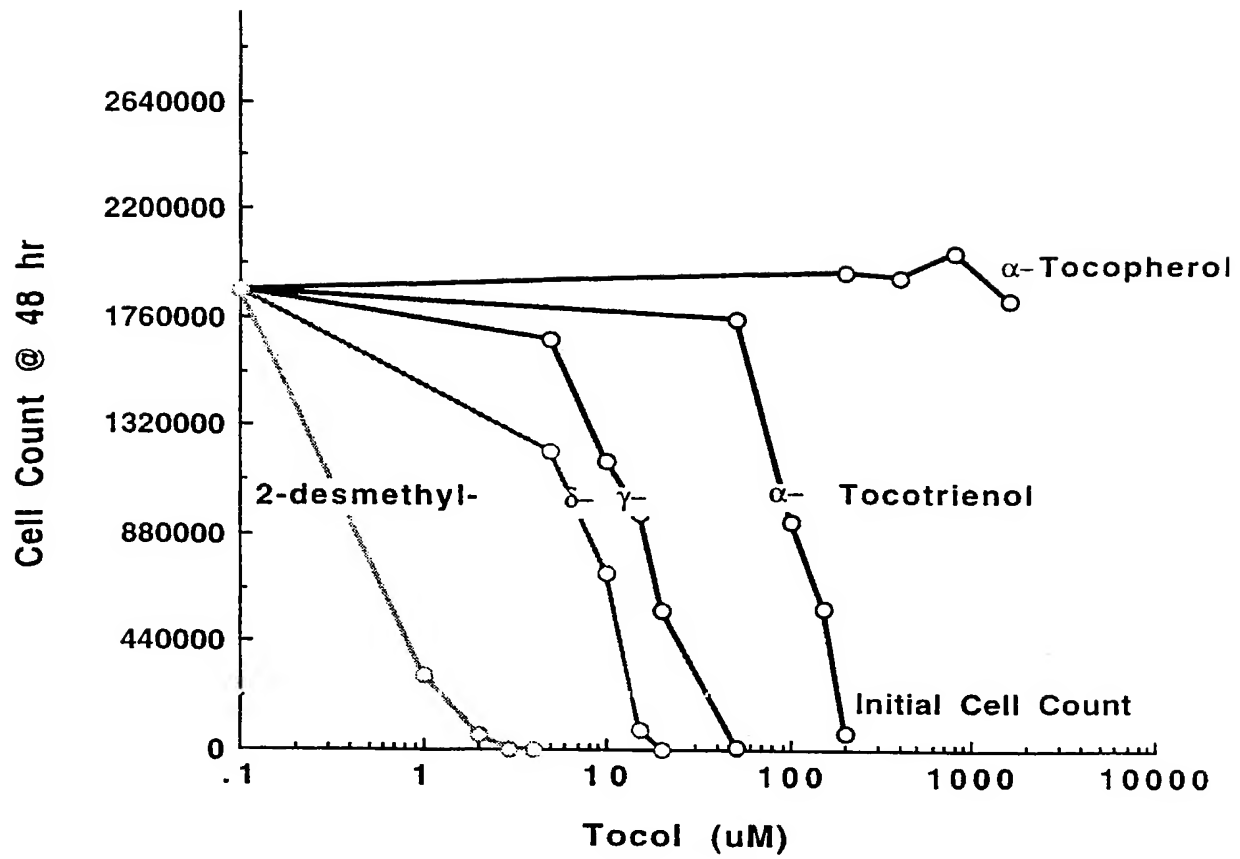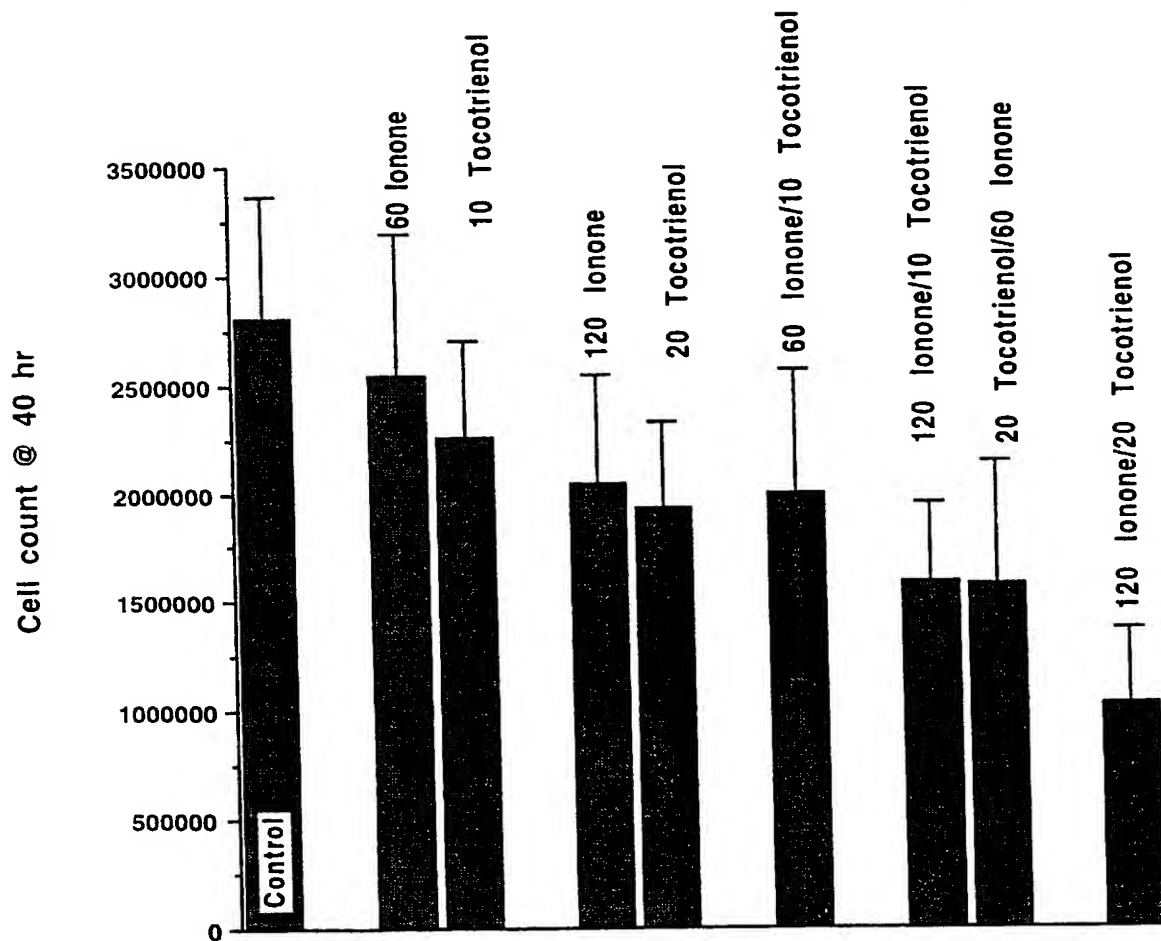between 200 mg and 400 mg and of statin is between 150 mg and
300 mg.

FIG. 1

FIG. 2A
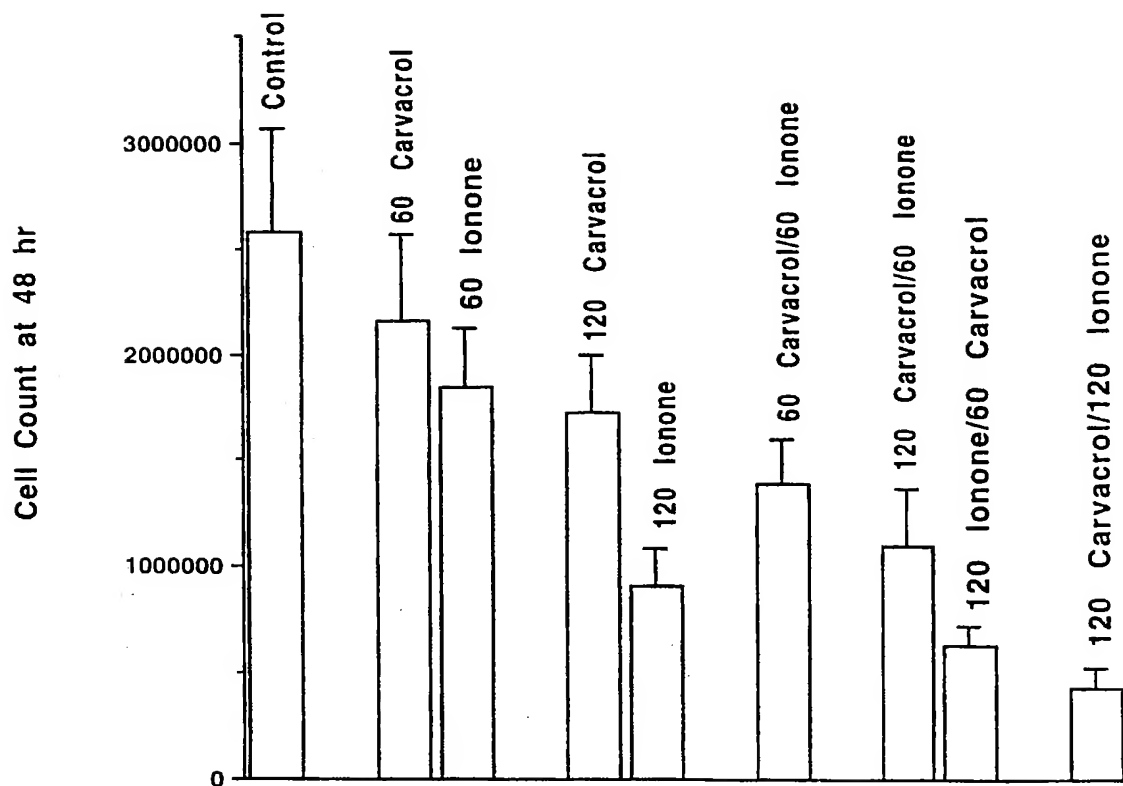
FIG. 2B
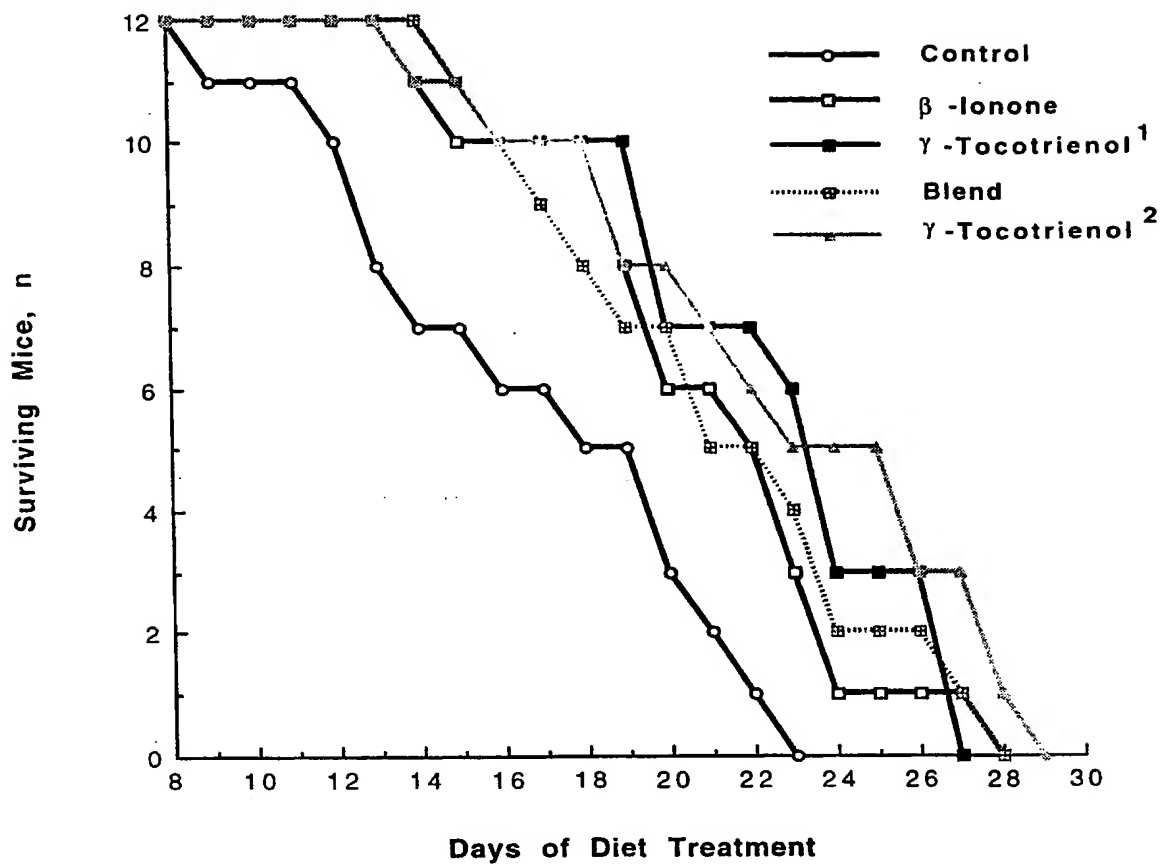
FIG. 3

INTERNATIONAL SEARCH REPORT

	Int 'ional Application No PCT/US 98/03470

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K31/355   A61K31/35

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K   A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ELSON: "Novel Lipids and Cancer: Isoprenoids and Other Phytochemicals" ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, vol. 399, 1996, pages 71-86, XP002067388	1,2,6-9, 11-15, 19,20
A	see page 71; figures 1-4 see page 75-76; table 1 see page 78, paragraph 3-4 see page 79, paragraph 3 ---	5,10,18
X	US 5 591 772 A (LANE RONALD H ET AL) 7 January 1997 see column 3, line 35-44; examples 19,20 see column 17, line 62 - column 18, line 7; claims 1,12,16 --- -/--	1,3,6, 10-14, 16,20-23

X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 1998	0 2. 07. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kanbier, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 20183 A (ZEAGEN INC) 14 October 1993 see page 3, line 11-13; claims 13,15 see page 10, line 18-27 see page 11, line 1-20 ---	14,17
X	WO 93 16690 A (WARNER LAMBERT COMPANY) 2 September 1993 see column 16, line 20-34; claims 1,3,6 see column 54, line 7-11 ---	14,16,20
A	US 5 605 930 A (SAMID DVORIT) 25 February 1997 see column 4, line 56 - column 5, line 30 see column 10, line 4-9 see column 20, line 3-11 ---	1,3,4, 10, 12-14, 16,17
A	WO 93 23034 A (US ARMY) 25 November 1993 see page 4, line 18-23; claims 1,2,11,15,20,22 ---	1,3,10, 12,13
A	EP 0 382 075 A (BASF AG) 16 August 1990 see column 1, line 45-50; claims 1,8 see column 4, line 15-35 ---	1,2,4,5, 9,12,13
A	US 5 376 361 A (PERRICONE NICHOLAS V) 27 December 1994 see column 7, line 28-32; claim 1 -----	1-3,5-7, 12,13

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/03470

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Although claims 1-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5591772	A	07-01-1997	AU	670557 B	25-07-1996
			AU	3150293 A	15-06-1993
			CA	2124086 A	27-05-1993
			CN	1073357 A	23-06-1993
			EP	0543417 A	26-05-1993
			JP	7504887 T	01-06-1995
			MX	9206735 A	01-05-1993
			NO	941929 A	13-07-1994
			WO	9309777 A	27-05-1993
WO 9320183	A	14-10-1993	AU	3919193 A	08-11-1993
			US	5422247 A	06-06-1995
WO 9316690	A	02-09-1993	AU	3315397 A	30-10-1997
			AU	4275793 A	13-09-1993
			CA	2129732 A	02-09-1993
			EP	0627917 A	14-12-1994
			JP	7505145 T	08-06-1995
			MX	9301014 A	01-09-1993
			US	5633285 A	27-05-1997
			ZA	9301297 A	27-06-1994
US 5605930	A	25-02-1997	US	5635532 A	03-06-1997
			AU	7973794 A	04-05-1995
			CA	2173976 A	20-04-1995
			EP	0725635 A	14-08-1996
			JP	9506079 T	17-06-1997
			WO	9510271 A	20-04-1995
			US	5708025 A	13-01-1998
			US	5712307 A	27-01-1998
			US	5654333 A	05-08-1997
			US	5661179 A	26-08-1997
			US	5710178 A	20-01-1998
			US	5635533 A	03-06-1997
			ZA	9407964 A	06-03-1996
			AU	2804592 A	21-05-1993
			CA	2108963 A	22-04-1995
			EP	0567613 A	03-11-1993
			NZ	244850 A	29-01-1997
			WO	9307866 A	29-04-1993

Form PCT/ISA/210 (patent family annex) (July 1992)

page 1 of 2

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5605930	A		ZA	9208140 A	21-04-1994
WO 9323034	A	25-11-1993	AU	683839 B	27-11-1997
			AU	4244393 A	13-12-1993
			CA	2135312 A	25-11-1993
			EP	0642341 A	15-03-1995
			JP	8503451 T	16-04-1996
EP 0382075	A	16-08-1990	DE	3903991 A	30-08-1990
			AU	617923 B	05-12-1991
			AU	4926490 A	16-08-1990
			CA	2007933 A	10-08-1990
			DE	59005487 D	01-06-1994
			DK	382075 T	24-05-1994
			JP	3197455 A	28-08-1991
			MX	9203498 A	01-08-1992
			US	5041648 A	20-08-1991
US 5376361	A	27-12-1994	US	5545398 A	13-08-1996

Form PCT/ISA/210 (patent family annex) (July 1992)